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Invited review: Whey proteins as antioxidants and promoters of cellular antioxidant pathways

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ABSTRACT

Oxidative stress contributes to cell injury and aggravates several chronic diseases. Dietary antioxidants help the body to fight against free radicals and, therefore, avoid or reduce oxidative stress. Recently, proteins from milk whey liquid have been described as antioxidants. This review summarizes the evidence that whey products exhibit radical scavenging activity and reducing power. It examines the processing and treatment attempts to increase the antioxidant bioactivity and identifies 1 enzyme, subtilisin, which consistently produces the most potent whey fractions. The review compares whey from different milk sources and puts whey proteins in the context of other known food antioxidants. However, for efficacy, the antioxidant activity of whey proteins must not only survive processing, but also upper gut transit and arrival in the bloodstream, if whey products are to promote antioxidant levels in target organs. Studies reveal that direct cell exposure to whey samples increases intracellular antioxidants such as glutathione. However, the physiological relevance of these in vitro assays is questionable, and evidence is conflicting from dietary intervention trials, with both rats and humans, that whey products can boost cellular antioxidant biomarkers.

Key words: whey products, whey proteins, bioactive peptides, antioxidant activity, oxidative stress

INTRODUCTION

Within each cell of the body, metabolic processes generate free radicals, and antioxidant systems are in place to effectively disarm them. However, this homeostatic balance can be altered due to excess free radical production, antioxidant depletion, or both. When the controls fail, cells are exposed to high levels of free radicals [reactive oxygen (**ROS**), reactive nitrogen, or

reactive sulfur species]. Oxidative stress ensues, leading to cell injury such as protein and lipid peroxidation, DNA strand breakage, racemization or decarboxylation of AA, enzyme dysfunction, and oxidative breakdown of carbohydrates (d'Ischia et al., 2006; Li et al., 2015). Sustained oxidative stress is considered a causative agent of neurodegenerative disorders (Gilgun-Sherki et al., 2001; Klein and Ackerman, 2003), cancer (Waris and Ahsan, 2006), liver injury (Li et al., 2015), aging (Lee et al., 2004), and appears to aggravate diabetes (Rochette et al., 2014), cystic fibrosis (Galli et al., 2012), chronic pancreatitis (Zhou et al., 2015), and cardiovascular disease (Sugamura and Keaney, 2011; Lönn et al., 2012). Cells protect themselves from oxidative damage by (1) prevention, (2) repair, (3) antioxidant production, or (4) uptake of dietary antioxidants or their precursors (Valko et al., 2007; Niki, 2010). Endogenous antioxidants include the intracellular enzymes superoxide dismutase (**SOD**) and catalase (**CAT**). The metal-binding enzyme, SOD, converts superoxide anion to hydrogen peroxide plus oxygen, whereas CAT converts hydrogen peroxide to water (Weydert and Cullen, 2010). The cytosolic Cys tripeptide, γ -glutamyl-cysteinyl-glycine, reduces hydroperoxides to alcohols and hydrogen peroxide to water by converting from its reduced (**GSH**) to its oxidized form. Well-documented dietary antioxidants include ascorbic acid (vitamin C), α -tocopherol (vitamin E), polyphenols, and carotenoids (Fiedor and Burda, 2014).

Recently, dairy proteins obtained from whey have received considerable attention for their antioxidant bioactivity (Bayram et al., 2008; Haraguchi et al., 2011; Zhang et al., 2012). Bovine whey proteins (**WP**) are widely used in various foods for their nutritional, health-promoting, and functional values (Ramos et al., 2017). Bovine liquid whey is produced by enzymatic treatment of milk (sweet whey) or addition of organic acids or minerals (acid whey) with the precipitation and removal of casein (Yadav et al., 2015). Bovine WP account for 11 to 14.5% of dry whey; the other components of bovine whey powder are lactose (63–75%), fat (1–1.5%), minerals (8.2–8.8%), and vitamins (A, C, E,

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and B groups; Miller et al., 2006; Yadav et al., 2015). The protein component of whey provides a complete protein source and is rich in sulfur-containing AA (1.7%; Fox et al., 2015) and in branched-chain AA (26%; Ha and Zemel, 2003; Paul, 2009). It is composed of β -LG (50–60%), α -LA (15–25%), BSA (6%), lactoferrin (<3%), and immunoglobulins (<10%; de Wit, 1998; Madureira et al., 2007; Le Maux et al., 2014). Beta-lactoglobulin is a small globular protein, composed of 162 AA with a molecular weight (M_W) of approximately 18,300 g/mol (Rade-Kukic et al., 2011). It contains all 20 EAA and is a rich source of sulfur. From a GSH precursor perspective, it has 5 Cys residues, 4 of them involved in disulfide bonds with the remaining 1 having a free reactive thiol group (Le Maux et al., 2014). Alpha-lactalbumin is a small protein with a M_W of 14,200 g/mol consisting of 123 AA arranged in a single peptide chain (Konrad and Kleinschmidt, 2008). It has 8 Cys as 4 disulfide bonds and, therefore unlike β -LG, has no free thiol group (Konrad and Kleinschmidt, 2008; Pepe et al., 2013). Bovine serum albumin is composed of 583 AA with a M_W of 66,430 g/mol (Hirayama et al., 1990). It contains 35 Cys groups making 17 disulfide bonds in addition to a free Cys which can facilitate intramolecular disulfide interactions (Madureira et al., 2007). Lactoferrin (M_W 80,000 g/mol) is an iron-binding monomeric globular glycoprotein (Wakabayashi et al., 2006) that contains 708 AA, of which 34 are Cys and all of which participate in disulfide bonds (Marshall, 2004). In addition, each lactoferrin monomer can bind 2 Fe^{3+} ions, with a binding affinity of 10 to 20 M (Baker and Baker, 2004); this iron-binding capacity is likely to contribute to its antioxidant potential (Baker and Baker, 2004; Kim et al., 2013). Bovine WP also contains dilute concentrations of immunoglobulins [Ig_A , Ig_M , and Ig_G (Ig_{G1} and Ig_{G2})]. These are quaternary structure molecules, either monomers or polymers with 4 chains, consisting of 2 light polypeptide chains (M_W 25,000 g/mol) and 2 heavy chains (M_W between 50,000–70,000 g/mol) linked by disulfide bonds (Madureira et al., 2007). Several bovine whey products are produced commercially (Table 1) and differ primarily in protein content and lactose concentration.

DO WHEY PRODUCTS SHOW ANTIOXIDANT ACTIVITY IN VITRO?

The antioxidant potential of WP has been assessed by different in vitro methodologies: 1,1-diphenyl-2-picrylhydrazyl (**DPPH**) radical assay, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (**ABTS**) assay, ferric-reducing antioxidant power (**FRAP**), and oxygen radical absorbance capacity (**ORAC**). Table 2 details the most recent studies of the antioxidant activity of whey products after processing, enzymatic hydrolysis, or both. Other noteworthy studies have been reviewed previously (Power et al., 2013; Brandelli et al., 2015). The WP antioxidant activity was shown to be dose-dependent (20–100 mg/mL) by the DPPH assay, which measures the ability of a compound to scavenge the DPPH radical (Gad et al., 2011). Hydrolysis of pre-heated whey protein isolate (**WPI**) with the enzyme subtilisin (EC 3.4.21.62), a nonspecific endopeptidase purified from *Bacillus licheniformis* and commercially available as Alcalase (Novozymes A/S, Bagsvaerd, Denmark), with specific activities ranging from 0.6 to 2.5 U/g, increased its DPPH scavenging activity from 11.4 to 62.9% (Peng et al., 2010). Hydrolysates of whey protein concentrate (**WPC**) produced by subtilisin also showed significantly greater inhibition than WP hydrolysates (**WPH**) produced by other microbial enzymes (Dryáková et al., 2010; Lin et al., 2012; O'Keeffe and FitzGerald, 2014). Dryáková et al. (2010) demonstrated 35.5% greater inhibition of the ABTS radical with WPC hydrolyzed by subtilisin rather than by the enzymes as bacillolysin [EC 3.4.24.28, commercial source Neutrase (Novozymes A/S, Bagsvaerd, Denmark)] or Protamex (EC 3.4.21.14, Novozymes A/S). The WPC hydrolyzed by subtilisin also showed more ferric-reducing power (0.55 mM $FeSO_4$ equivalents) than trypsin (EC 3.4.21.4), pepsin (EC 3.4.23.1), or leucyl aminopeptidase (EC 3.4.11.1, commercial source Flavourzyme, Novozymes A/S) hydrolysates (0.35 mM $FeSO_4$ equivalents, $P < 0.05$). This activity was further increased by heat treatment (95°C, 5–10 min) of WPC before hydrolysis (Lin et al., 2012). However, Adjonu et al. (2013) observed that heat pretreatment (80°C, 15 min) did not improve antioxidant activity of WPI hydrolysates from pepsin [nonheated WPH = 0.32 ± 0.03 μ mol of Trolox equivalents (**TE**)/mg of protein; heated WPH = 0.30 ± 0.03 μ mol of TE/mg of protein] or chymotrypsin (EC 3.4.21.1; nonheated WPH = 0.27 ± 0.04 μ mol of TE/mg of protein; heated WPH = 0.31 ± 0.02 μ mol of TE/mg of protein). In Adjonu et al. (2013), ORAC methodology was employed, which scavenges peroxy radicals and compares levels to the vitamin E analog, Trolox.

Table 1. Composition of commercial whey protein products

Whey products	Composition		
	Protein (%)	Fat (%)	Lactose (%)
Whey protein concentrate	34–80	1–7	4–52
Whey protein isolate	90–95	0.5–1	0.5–1
Hydrolyzed whey protein	80–90	0.5–8	0.5–10

In an effort to increase antioxidant properties of peptides from WP, Le Maux et al. (2016) altered the hydrolysis conditions (pH, enzyme type, reaction time, and temperature). Hydrolysis of WP (81% protein) test samples with papain (EC 3.4.22.2) at a constant pH of 7.0 gave significantly higher ORAC values (285.32 ± 36.71 μmol of TE/g of powder) than those obtained from hydrolysates generated under noncontrolled pH conditions (192.54 ± 42.61 μmol of TE/g of powder, $P < 0.05$). To characterize the functional fraction of whey, WPC was hydrolyzed by several enzymes and the resultant hydrolysates were fractioned by size using gel or membrane filtration (Peng et al. 2009; Önay-Ucar et al. 2014; Tarango-Hernández et al. 2015). The ORAC results showed that fractions containing peptides with smaller molecular weight (subtilisin hydrolysate 1-kDa permeate = 0.91 μmol of TE/mg of powder) exhibited more antioxidant activity than those fractions containing larger peptides (subtilisin hydrolysate 5-kDa permeate = 0.75 μmol of TE/mg of powder; O'Keeffe and FitzGerald, 2014).

Mass spectrometry analysis revealed the AA sequences of peptides in antioxidant fractions (Table 3). Several peptides from β -LG were identified in antioxidant fractions produced by enzymatic hydrolysis of whey products. Many of these peptides occurred within 3 location hotspots (42–61, 77–110, and 123–135 AA). Interestingly 125 to 135 AA contains the iron-binding peptide TPEVDDEALEK (Cruz-Huerta et al., 2016). Bertucci et al. (2015) also discovered several α -LA peptides in fractions exhibiting antioxidant activity. In this case, peptides from location 15 to 23 AA were frequently identified. To date none of these peptides have been synthesized and tested in DPPH, ABTS, ORAC, or FRAP assays for antioxidant activity. The free AA present in these fractions, some of which may contribute to the antioxidant activity (e.g., Trp, Phe, Tyr, Cys, and His) have also not been described. In contrast (Table 3), Hernández-Ledesma et al. (2005) identified several peptides and AA from a 3-kDa permeate of the β -LG hydrolyzed by Corolase PP (AB Enzymes, Darmstadt, Germany). Three peptides, MHIRL, YVEEL, and WYSLAMAASDI, were synthesized and exhibited antioxidant activity by ORAC (MHIRL = 0.306 μmol of TE/ μmol of peptide; YVEEL = 0.799 μmol of TE/ μmol of peptide; and WYSLAMAASDI = 2.621 μmol of TE/ μmol of peptide). In particular, the antioxidant activity of WYSLAMAASDI is comparable to the synthetic antioxidant butylated hydroxyanisole (2.43 μmol of TE/ μmol pure compound), but 1.7 to 4 fold lower than the plant polyphenols catechin and quercetin (14.9 and 10.5 μmol of TE/ μmol of compound, respectively; Dávalos et al., 2004). Additionally, Hernández-Ledesma et al. (2007) identified 3 synthetic peptides derived

from β -LG (19–24 AA; WY, WYS, and WYSLAM) that exhibited ORAC values [4.45 (WYS) to 7.67 (WY) μmol of TE/ μmol of peptide] higher than equimolar mixtures of their corresponding free AA. Nongonierma and Fitzgerald (2013) identified a synthetic dipeptide WC present in α -LA (60–61 AA) and lactoferrin (8–9 and 347–348 AA) that exhibited 50% DPPH scavenging capacity at 0.26 mM, equivalent to 17.2 nM Trolox. Moreover, purified peptides, LDQW and INYW, derived from thermolysin (EC 3.4.24.27) hydrolysis of α -LA were capable of a 100% ABTS radical inhibition at 2.5 μM (Sadat et al., 2011).

In addition to processing, whey origin may also play a role in antioxidant activity. Salami et al. (2010) hydrolyzed camel WP with either chymotrypsin, trypsin, proteinase K (EC 3.4.21.64), or thermolysin. Camel liquid whey is rich in α -LA and lysozyme, but lacks β -LG. Camel WP exhibited 40% higher antioxidant activity by ABTS than bovine WP (Salami et al., 2010). In addition, sheep WP was found to be more active against the DPPH radical, requiring 3.1 ± 0.09 mg/mL to inhibit 50% of the radical compared with 8.2 ± 0.77 mg of bovine WP/mL (Kerasioti et al., 2014). It also exhibited greater iron-reducing power than bovine WP, although, in this case, ABTS data was similar (Kerasioti et al., 2014). Indeed, conflicting data between different radical scavenging methods (ORAC, DPPH, ABTS) is common to the majority of studies (Adjonu et al., 2013; Kerasioti et al., 2014), indicating the need to perform several antioxidant assays to be confident of results. Interestingly, other components in milk appear to synergistically enhance the antioxidant activity of WP. Zulueta et al. (2009) showed higher ORAC values for pasteurized milk ($13,935$ μM TE) than from whey obtained after casein precipitation of pasteurized milk ($1,078$ μM TE). In this regard, Conway et al. (2013) observed that hydrolysates from buttermilk protein (54.6% protein content) were more effective ($P < 0.05$) at scavenging free radicals than those from WPC (74.5% protein content). The ORAC values were $1,319.6 \pm 46.7$ μmol TE/g of protein for buttermilk compared with 782.5 ± 34.8 μmol TE/g of protein for WPC. Although buttermilk is unlikely to contain large quantities of WP, analysis revealed 4 β -LG peptides (VAGTWY, TKIPAVFK, IPAVF, and VLVLDTDYK) that were proposed to contribute to the antioxidant activity (Conway et al., 2013).

Recently, whey products have been added to nutritional beverages to boost their antioxidant capacity. Supplementation of a lemon drink with 1% WP hydrolyzed by subtilisin increased the antioxidant activity of the beverage from 0.75 to 7.79 mmol of TE/L (Athira et al., 2014). In addition, a flavored milk beverage fortified with 1 or 2% WPH from different enzymes

Table 2. Effect of processing treatments and enzyme hydrolysis on the antioxidant activities of whey protein isolate, whey protein concentrate, and whey protein

Product ¹	Heat treatment	Hydrolysis/filtration/other ²	Antioxidant assay ³	Results ⁴	Reference
WPI (95% protein)	Preheating 95°C, 5 min	Subtilisin (pH 8.5, 65°C, 1, 2, 3, 4, 5, and 8 h)	Inhibition liposomes peroxidation TBARS FRAP Metal-chelating activity DPPH	Hydrolysis ↑ AOX (DPPH inhibition: WPI = 11.4%, 5h-WPH = 62.9%)	Peng et al. (2010)
WPI	Preheating 85°C, 15 min	Papain (pH 8.0, 35–55°C, 2–6 h)	Reducing power assay DPPH	<AOX at 3.6 h, 45.7°C (DPPH = 31.36%)	Zhidong et al. (2013)
WPI	Unheated or Preheated (80°C, 15 min)	Pepsin (pH 2.6, 37°C) Trypsin (pH 7.8, 37°C) Chymotrypsin (pH 7.8, 37°C) 12 or 24 h	ABTS ORAC	Hydrolysis ↑ AOX (WPH trypsin = 0.32, WPI = 0.08 μmol of TE/mg of protein) No differences between enzymes, time or preheating	Adjou et al. (2013)
Native and pressurized WPI (1 mg/mL)		Pressure treatment (1 cycle of 550 MPa) followed by SGID: pepsin (pH 1.9, 37°C, 0.25 h), trypsin, chymotrypsin and peptidase (pH 7.4, 37°C, 1.0 h), membrane filtration (10 kDa permeate)	FRAP	SGID pressurized WPI showed 21% more AOX activity than SGID native WPI	Iskandar et al. (2015)
WPI		Papain (pH 7.0, 65°C, 0–5 h) Pepsin (pH 2.0, 37°C, 0–5 h) Subtilisin (pH 8.3, 55°C, 0–5 h)	Ferric reducing power	Papain AOX results were time-dependent. No change in AOX activity of subtilisin or pepsin hydrolysates	Mohan et al. (2015)
WPI (≥90% protein)	Preheated (85°C, 5 h, pH 2.0)	Corolase N (pH 7.7, 55°C, 5 h)	DPPH Reducing power	Hydrolysis and heat ↑ AOX activity (Scavenging activity: WPI = 13%, WPH = 60%)	Mohammadian and Madadlou (2016)
WPI (90% protein)	Preheating 80°C, 7 min	Pepsin (pH 2.0, 37°C, 0–12 h)	Ferric reducing power	Maximum AOX activity at 6 h	Nourbakhsh et al. (2017)
WPC (80% protein)			DPPH Ferrous-chelating activity	WPC AOX activity dose-dependent (20–100 mg/100 mL)	Gad et al. (2011)
WPC	Unheated or Preheated (95°C, 5 or 10 min)	Pepsin (pH 2.0, 37°C, 2 h) Trypsin (pH 8.0, 37°C, 2 h) Subtilisin (pH 9.0, 50°C, 2 h) Leucyl aminopeptidase (pH 7.0, 50°C, 2 h)	Total AOX activity FRAP DPPH	Hydrolysis ↑ AOX activity (subtilisin = 62% DPPH inhibition > trypsin or pepsin)	Lin et al. (2012)
WPC	Preheating 90°C, 5 min	Pepsin (pH 1.5, 37°C) followed by trypsin (pH 7.6, 50°C) for 1.5 h	DPPH Superoxide anion radical scavenging activity Ferric reducing power ORAC	WPH dose dependent AOX activity (0–10 mg/mL)	Zhang et al. (2012)
WPC, commercial WPH (DH = 32%; DH = 45%)		SGID: Pepsin (pH 2.0, 37°C, 1.5 h) followed by Corolase PP (pH 7.5, 37°C, 2.5 h)		WPH AOX activity 3 - 6 fold > WPI. SGID ↑ AOX WPC (WPC = 13,662; SGID WPC = 36,605 μmol of TE/100 g powder)	Power-Grant et al. (2015)
WPC (4% protein)	Preheating 50–54°C, 10 min	Protamex, subtilisin, or both (pH 7.0; 0.5, 1, 1.5 h; 45, 50, 55°C)	ABTS ORAC	WPC = 2.83 mM TE WPH Protamex = 4.27 mM TE WPH both enzymes = 6.33 mM TE	Torkova et al. (2016)
WPX (11% protein)			DPPH	WP 72.15% scavenging activity	Ashoush et al. (2013)
WPX		Leucyl aminopeptidase Subtilisin Protease from <i>Aspergillus oryzae</i> Optimal temperature and pH, 4 h	ORAC DPPH	Hydrolysis ↑ AOX activity of WP Hydrolysates by protease from <i>Aspergillus oryzae</i> > ORAC values (172.11 μmol of TE/g) and DPPH inhibition (69.53%)	de Castro and Sato (2014)
Sweet and acid WPC		Polymerization by glycation (pH 7.0 and 9.0) Hydrolyzed by biomass of <i>Bacillus subtilis</i> (pH 7.0, 50°C, 24 h)	ABTS	Hydrolysis and glycation ↑ AOX activity (acid WPC = 55%, WPH = 85%, WPC glycated = 75%, WPH glycated = 95%)	Ortega et al. (2015)

Continued

Table 2 (Continued). Effect of processing treatments and enzyme hydrolysis on the antioxidant activities of whey protein isolate, whey protein concentrate, and whey protein

Product ¹	Heat treatment	Hydrolysis/filtration/other ²	Antioxidant assay ³	Results ⁴	Reference
MPC, commercial WPH (DH = 32%, 78% protein; DH = 45%, 75% protein)			ORAC	WPH45 = 77,691 μ mol of TE/100 g powder > WPH32 = 37,391 μ mol of TE/100 g powder > MPC 15,678 μ mol of TE/100 g powder	Power-Grant et al. (2016)
WPX (81% protein)		Papain (pH 7.0, 6.3 and without pH control, 50°C, 3 h)	ORAC	Higher AOX at constant pH 7.0 (WP = 71.52 μ mol of TE/g protein, WPH = 285.32 μ mol of TE/g protein)	Le Maux et al. (2016)

¹WPI = whey protein isolate; WPC = whey protein concentrate; WPH = whey protein hydrolysate; DH = degree of hydrolysis; WPX = whey protein, type not specified; MPC = milk protein concentrate.

²SGID = simulated gastrointestinal digestion; Corolase N and Corolase PP (AB Enzymes, Darmstadt, Germany); Protamex (Novozymes A/S Bagsvaerd, Denmark).

³TBARS = thiobarbituric acid reactive substances; FRAP = ferric-reducing antioxidant power; DPPH = 1,1-diphenyl-2-picrylhydrazyl radical; ABTS = 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); ORAC = oxygen radical absorbance capacity.

⁴AOX = antioxidant; TE = Trolox equivalents.

[leucyl aminopeptidase, subtilisin, or Corolase PP (AB Enzymes)] increased the ABTS radical inhibition of the beverage by 21 and 33%, respectively. Interestingly, adding intact WPC (1–2%) to the beverage did not alter ABTS values (Mann et al., 2015). A polyphenol rich beverage [chlorogenic acid (0.01%) or catechin (0.01%)], thermally treated (121°C, 10 min) at pH 3.7, exhibited ABTS values of 0.45 to 1.22 mM TE/L respectively. However, the addition of WP (0.2%; ABTS = 0.45 mM TE/L) to this model beverage did not result in additive antioxidant activity, although ABTS results were higher than the beverage with polyphenol values alone (0.90–1.77 mM TE/L; He et al., 2015). Indeed, addition of WP (0.5, 2.0, 4.0, or 6.0%) did not significantly change ($P > 0.05$) the antioxidant activity of another beverage with 0.0032% lutein, a carotenoid antioxidant (Rocha et al., 2017). Interestingly, the combination of WPC and the algae *Spirulina platensis*, rich in carotenoids, tocopherol, and phycocyanin, showed less antioxidant power (125 TE mg/L of sample) than *Spirulina platensis* (100 mg/100 mL) alone (170 TE mg/L of sample), which indicates that whey products can exert an antagonistic effect on the antioxidant activity of other compounds (Gad et al., 2011).

How WP compare in terms of their antioxidant activity to other proteins and known antioxidant compounds has been investigated (Dávalos et al., 2004; Hernández-Ledesma et al., 2007; Castro and Sato, 2014). Intact WP showed significantly lower DPPH radical inhibition ($17.13 \pm 2.33\%$) than soy protein isolate ($27.18 \pm 0.15\%$) or egg white protein ($33.39 \pm 0.26\%$; Castro and Sato, 2014), although the purity of each protein

was not described. Interestingly, no significant differences in DPPH inhibition were found between WP ($29.81 \pm 0.48\%$) and egg ($31.50 \pm 0.24\%$) hydrolysates using leucyl aminopeptidase (Castro and Sato, 2014). In contrast, ORAC values for WPH were lower ($160.72 \pm 26.26 \mu$ mol of TE/g) than results obtained for their counterparts from egg ($546.45 \pm 55.75 \mu$ mol of TE/g) or soy ($1,157.18 \pm 134.66 \mu$ mol TE/g), which again underlines the inconsistencies across antioxidant assays (Castro and Sato, 2014). It is noteworthy that 100 g of WPC (79.0% protein) results in ORAC values of $13,662 \pm 1,018 \mu$ mol of TE (Power-Grant et al., 2015), whereas 100 g of concentrated green tea extract results in 758,000 μ mol of TE (de la Luz Cádiz-Gurrea et al., 2014). However, as a protein, WP can be added to foods at concentration of 22.2% (Chavan R. S. et al., 2015), whereas green tea extract is usually added to foods at concentrations less than 0.04% (Maruyama et al., 2017).

CAN WHEY PRODUCTS BOOST INTRACELLULAR ANTIOXIDANT DEFENSES IN VITRO?

According to the Swedish Agency for Health Technology Assessment and Assessment of Social Services and cited by the World Health Organization, boosting antioxidants capabilities (GSH, CAT, and SOD) in cells by the diet will achieve long life and well-being (SBU, 1997). At cellular levels, GSH (1) directly scavenges free radicals (for example hydroxyl radicals); (2) is a substrate for the antioxidant enzymes glutathione peroxidase and glutathione transferase; (3) facilitates

Table 3. Peptides identified in antioxidant bovine whey fractions

Product ¹	Hydrolysis/filtration/other pretreatments ²	Antioxidant assay ³	Peptides in antioxidant fractions	Reference
WPC	Thermolysin (pH 8.0, 80°C, 8 h) followed by membrane filtration 3 kDa cut-off	ORAC	β-LG: f(58–61) LQKW, f(95–101) LDTDYKK	Contreras et al. (2011)
Fresh whey	Subtilisin (pH 7.0–9.0, 50–70°C, 2–8 h) followed by membrane filtration 3 kDa cut-off	ABTS	β-LG: f(96–100) DTDYK, f(94–100) VLDTDYK, f(123–131) VRTPEVDDE, f(122–131) LVRTPEVDDE, f(124–131) RTPEVDDEALE, f(123–134) VRTPEVDDEALE, f(122–134) LVRTPEVDDEALE	Athira et al. (2014)
WPC	Subtilisin (pH 8.5, 65°C, 5 h) Leucyl aminopeptidase (pH 6.5, 50°C, 8 h) Corolase PP (pH 7.5, 45°C, 7 h) followed by membrane filtration: 3 kDa cut-off	ABTS	Corolase PP fraction: β-LG: f(50–56) PEGDLEI, f(43–49) VEELKPT, f(43–51) VEELKPTPE, f(125–135) TPEVDDEALEK	Mann et al. (2015)
WPC	Serine peptidase from <i>Machura pomifera</i> (pH 6.5, 45°C, 0–3 h)	ABTS	α-LA: f(5–12) KCEVFREL, f(6–13) CEVFRELK, f(13–19) KDLKGYG, f(15–22) LKGYGGVS, f(16–23) KGYGGVSL, f(32–38) HTSGYDT, f(40–57) AIVQNDSTEYGLFQINN, f(82–88) DDDLTDD, f(104–113) WLAHKALCSE β-LG: f(10–28) LDQKVAGTWYSLAASD, f(11–29) DIQKVAGTWYSLAASDI, f(42–47) YVEELK, f(52–61) GDLEILLQKW, f(55–61) EILLQKW, f(56–62) ILLQKWE, f(77–87) KIPAVFKIDAL, f(89–96) ENKVLVLD, f(95–110) LDTYKKYLLFCMENS, f(150–160) SFNPTQLEEQC, f(151–162) FNPTQLEEQCHI β-LG: f(27–38) DIQKVAGTWYSL, f(33–38) GTWYSL, f(39–48) AMAASDISLL, f(40–48) MAASDISLL, f(61–73) ELKPTPEGDLEIL, f(87–98) IIAEKTIPAVF, f(112–121) DTDYKKYLLF, f(165–172) LSFNPTQL β-LG: f(19–29) WYSLAASDI, f(42–46) YVEEL, f(145–149) MHIRL β-LG: f(19–20) WY, f(19–21) WYS, f(19–22) WYSL, f(19–23) WYSLA, f(19–24) WYSLAM α-LA: f(101–104) INYW, f(115–118) LDQW	Bertucci et al. (2015)
β-LG	Subtilisin (pH 7.0, 50°C, 2 h) under high-hydrostatic pressure	FRAP, iron chelating activity		Bandad et al. (2017)
β-LG	Corolase PP (pH 8.0, 37°C, 24 h)	ORAC		Hernandez-Ledesma et al. (2005)
Synthetic peptides		ORAC		Hernandez-Ledesma et al. (2007)
Purified peptides	Thermolysin (pH 7.2, 70°C, 15 min)	ABTS		Sadat et al. (2011)
Synthetic peptides		DPPH		Nongonierna and Fitzgerald (2013)
β-LG	Trypsin (pH 8.0, 37°C, 24 h)	ORAC	β-LG: f(19–20) WY, f(60–61) KW α-LA: f(25–26) EW, f(60–61) WC, f(103–104) YW, f(104–105) and f(118–119) WL BSA: f(133–134) FW	Power et al. (2014)
WPC	Corolase PP (pH 7.0, 50°C, 4 h) followed by membrane filtration: 0.65 kDa cut-off	ORAC	Lactoferrin: f(16–17) WF, f(22–23) WQ, f(24–25) WR, f(124–125) and f(466–467) GW, f(137–138) SW, f(138–139) WT, f(198–199) LW, f(448–449) and f(467–468) WN β-LG: f(15–20) VAGTWY β-LG: f(15–18) VAGT, f(24–26) MAA, f(71–74) IIAE	O'Keeffe et al. (2017)

¹WPC = whey protein concentrate, WPI = whey protein isolate, WPX = whey protein, type not specified.²Corolase PP (AB Enzymes, Darnstadt, Germany).³ORAC = oxygen radical absorbance capacity, ABTS = 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), FRAP = ferric-reducing antioxidant power, DPPH = 1,1-diphenyl-2-picrylhydrazyl radical.

transport of AA, specifically Cys, across the plasma membrane; (4) regenerates antioxidants (e.g., vitamins C and E) to their functional form; and (5) forms conjugates with toxic electrophilic compounds, catalyzed by glutathione transferase, which are excreted from cells (Pastore et al., 2003; Masella et al., 2005; Valko et al., 2007). Tseng et al. (2006) reported that the rat renal cell line, PC12, pretreated with WPC at 10 mg/L for 24 h before an ethanol stress, produced 59.4 mM GSH/mg of protein compared with 29.9 mM GSH/mg of protein ($P < 0.05$) for cells with no WPC pretreatment. This indicates whey products may offer a protective benefit to cells when stressed. In agreement, stressing myoblast cells C₂C₁₂ with 0.3 mM *tert*-butyl hydroperoxide (**t-BHP**) for 30 min decreased GSH levels by 31.5% compared with control, as measured by flow cytometry. These t-BHP stressed C₂C₁₂ when pretreated for 24 h with sheep WP at 1.56, 3.12, and 6.24 mg increased GSH levels 112.9, 118.0, and 138.0%, respectively, compared with levels of t-BHP-stressed cells (Kerasioti et al., 2014). In a recent study (Table 4), t-BHP was also used to stress human hepatocytes (HepG2) for 2 h after 24 h of WPC treatment (100 µg/mL; Pyo et al., 2016). The WPC treatment increased GSH levels (130%) from basal conditions and also recovered GSH levels from stressed cells (80%). In an attempt to identify which whey fraction is responsible for increasing GSH, O'Keeffe and FitzGerald (2014) used enzymatically hydrolyzed WPC to treat human umbilical vein endothelial cells (**HUVEC**) and GSH levels were monitored. The WPC was hydrolyzed by subtilisin, bacillolysins, Corolase PP (AB Enzymes), and leucyl aminopeptidase and the resulting peptide fractions were separated according to size using 0.2 µm, 10 kDa, 5 kDa, and 1 kDa cut-off membranes. Whey hydrolysate fractions by subtilisin, bacillolysins, Corolase PP, and leucyl aminopeptidase at 1 mg/mL significantly increased intracellular GSH in HUVEC cells ($P < 0.05$) after 48 h of incubation compared with HUVEC cells cultured in media alone. The 1-kDa permeate of hydrolysate from subtilisin treatment increased GSH levels by 153% in HUVEC cells compared with media alone ($P < 0.001$). Kent et al. (2003) incubated prostate epithelial cells (**RWPE-1**) for 48 h with (1) 0.5 mg/mL of hydrolyzed WPI, (2) 0.5 mg/mL of hydrolyzed casein, (3) 500 µM buthionine sulfoximine (GSH synthesis inhibitor), or (4) 500 µM *N*-acetylcysteine (GSH stimulant). *N*-Acetylcysteine increased GSH by 88% in RWPE-1 cells. Hydrolysates of WP with trypsin, chymotrypsin, and peptidase increased GSH by 64% compared with hydrolyzed casein-treated and control cells ($P < 0.05$). Interestingly, the 50% reduction of GSH levels in RWPE-1 cells by buthionine sulfoximine could not be reversed by co-treatment with WPH, but

could be reversed with *N*-acetylcysteine (Kent et al., 2003). Vilela et al. (2006) evaluated a combination of high hydrostatic pressure processing and low-M_w whey peptide fractions, but did not observe a boost in GSH levels in human tracheal epithelial cells (9HTEo cell line).

To investigate if WP increased CAT activity in cells, C₂C₁₂ muscle cells were treated with 0.1 to 0.4 mg/mL of WP (80.05% protein) for 24 h and then stressed with 0.75 mM H₂O₂ for 1 h (Xu et al., 2011). The CAT activity was significantly enhanced from 15.1 ± 0.7 to 23.7 ± 1.3 U/mg of total cellular protein ($P < 0.05$) by WPC. Similarly, CAT activity was increased 141% in HUVEC cells after a 48-h incubation with 1 mg/mL of 1-kDa permeate of WP hydrolyzed with Corolase PP compared with media alone ($P < 0.01$) (O'Keeffe and FitzGerald, 2014). In addition, CAT activity also increased in H₂O₂-stressed lung fibroblasts (MRC-5 cell line) after 24 h of treatment with 100 µg/mL of subtilisin WPH from 25 to 65 U/mg of protein (Kong et al., 2012).

The SOD activity was also determined in stressed C₂C₁₂ cells (Xu et al., 2011). Once again, cells were preincubated with WP (0.1–0.4 mg/mL) for 24 h, and then stressed for 1 h with 0.75 mM H₂O₂. Pretreatment with WP significantly increased SOD levels (11.7 ± 0.5 U/mg of protein) in stressed cells compared with cells that did not receive WP treatment (5.27 ± 0.41 U/mg of protein). In addition, WP also increased SOD activity in nonstressed cells from 13.4 ± 0.82 to 19.4 ± 0.6 U/mg of protein. Similarly, 24 h of pretreatment with subtilisin hydrolysates of WPI (20 µg of WPH/mL) increased SOD activity in H₂O₂-stressed lung fibroblasts compared with non-whey-treated cells by 248% (Kong et al., 2012).

The vast majority of experiments to date expose cells lines to whey test samples. The usual mode of delivery of whey products is via food consumption, so the physiological relevance of such experiments is questionable. Target cells will only be exposed to whey components arriving in the bloodstream from the gut. Bovine WP are easily and rapidly digested to individual AA in the gastrointestinal tract, showing maximum concentration of total AA in plasma at 69 min post-WPI consumption (Purpura et al., 2014). Indeed, WPI has a digestible indispensable AA score of 1.09 (Rutherford et al., 2015). Power-Grant et al. (2015) performed a simulated gastrointestinal digestion of intact WPC and then measured its antioxidant activity by ORAC. Gastric digestion of WPC increased its ORAC values by 2.5 fold compared with intact WPC. However, when the WPC was in a hydrolyzed form, gastric digestion resulted in a 22% decrease in ORAC values, which indicates that bioactivity of hydrolyzed whey samples was reduced

Table 4. Antioxidant activity of whey products determined by cell culture

Whey product ¹	Protein pretreatment: Hydrolysis/filtration/other ²	Cell line	Incubation time/stress	Antioxidant assay ³	Results	Reference
WPC (0.4 mg/mL)		C ₂ C ₁₂ (mice myoblast)	24 h followed by H ₂ O ₂ (0.75 mM, 1 h)	GSH CAT SOD TBARS GPx DNA oxidative damage AOX gene expression	After H ₂ O ₂ stress, WPC ↑ GSH, CAT, SOD, GPx; and HO-1 = NRF2 and NQO1 ↓ Lipid peroxidation and DNA damage	Xu et al. (2011)
WPI (4, 20 or 100 μg/mL), 2.8–40 kDa fraction	Preheating 95°C, 5 min Subtilisin (pH 8.5, 65°C, 5 h) Followed by gel filtration (>40, 2.8–40, 0.1–2.8, <0.1 kDa)	MRC-5 (human lung fibroblast)	24 h followed by H ₂ O ₂ (1 mM, 24 h)	AOX enzymes: SOD, CAT, GPx Cellular lipid oxidation inhibition	WPI (20 and 100 μg/mL), protected against induced-lipid peroxidation by increasing SOD, CAT, GPx	Kong et al. (2012)
WPC (0.05–0.4 mg/ mL)	Preheating 90°C, 5 min Pepsin (pH 1.5, 37°C) followed by trypsin (pH 7.6, 50°C) for 1.5 h	PC12 (rat pheochromocytoma)	2 h followed by H ₂ O ₂ (100 μM, 24 h)	Cell viability	WPI protected against H ₂ O ₂ cytotoxicity	Zhang et al. (2012)
Native and pressurized WPI (0–2 mg/mL)	Pressure treatment (1 cycle 550 MPa) followed by SGID (Pepsin (pH 1.9, 37°C, 0.5 h), trypsin and chymotrypsin (pH 7.4, 40°C, 1.5 h); 10 kDa permeate	Caco-2 (human colonic adenocarcinoma)	H ₂ O ₂ (0.25 mM, 1 h) with or without SGID whey (23 h)	Intracellular ROS FRAP	↓ ROS at 2 mg/mL: SGID native WPI = 32.5%, SGID pressurized WPI = 76.1% compared with stressed but non-WPI-treated cells FRAP activity of SGID WPI- treated cell medium > nontreated > stressed cell medium WPC did not ↑ GSH nor CAT All 1 kDa hydrolysates ↑ GSH All 5 kDa hydrolysates ↑ CAT unlike 0.2 μm Gene expression of GPX3, NADPH dehydrogenase quinone 1 and 2, Aldehyde dehydrogenase 3 family, member A1 ↑ with 5 kDa permeates of subtilisin and bacillolysins compared with baseline FRAP of SGID pressurized WPI- treated-cell 35% greater than control	Piccolomini et al. (2012)
WPC (1 mg/mL)	Subtilisin Bacillolysins Corolase PP Leucyl aminopeptidase General conditions: pH 7.0, 50°C, 4 h Followed by membrane filtration (0.2 μm, 5 and 1 kDa)	HUVECs (human umbilical vein endothelial)	48 h	GSH CAT Microarray analysis		O'Keefe and FitzGerald (2014)
Native and pressurized WPI (0–1 mg/mL)	Pressure treatment (1 cycle 550 MPa) followed by SGID: - Pepsin (pH 1.9, 37°C, 0.25 h) followed by trypsin, chymotrypsin and peptidase (pH 7.4, 37°C, 1.0 h). Then, membrane filtration (10 kDa permeate) Glucose-WPC conjugate	1HAEO (human tracheobronchial epithelial)	6 h	FRAP		Iskandar et al. (2015)
WPC (0.1 mg protein/ mL)		HepG2 (human hepatocytes)	24 h followed by <i>tert</i> - butylhydroperoxide (1 mM, 2 h)	GSH ROS production AOX gene expression	WPC and Glucose-WPC ↑ GSH (130%, 150%), ↓ ROS (140%, 120%) from control (100%) and stressed cells (GSH: 80%, ROS: 160%)	Pyo et al. (2016)

¹WPC = whey protein concentrate, WPI = whey protein isolate.²SGID = simulated gastrointestinal digestion; Corolase PP (AB Enzymes, Darmstadt, Germany).³GSH = glutathione, CAT = catalase, SOD = superoxide dismutase, TBARS = thiobarbituric acid reactive substances, GPx = glutathione peroxidase; AOX = antioxidant; ROS = reactive oxygen species; FRAP = ferric-reducing antioxidant power.

during gut transit (Power-Grant et al., 2015). To assess the antioxidant benefit to intestinal cells exposed to gastric-digested whey products, the intestinal epithelial cell line, Caco-2, was stressed with 0.25 mM H_2O_2 for 1 h and then treated with gastric-digested WPI (0–2 mg/mL) for 23 h. The ROS activity in Caco-2 cells were reduced by 32.5% when cells were treated with 2 mg/mL of gastric-digested WPI compared with ROS values from stressed cells (Piccolomini et al., 2012). Picariello et al. (2013) recently reported whey peptides that are bioavailable across the Caco-2 intestinal barrier model, postgastrointestinal digestion. Interestingly, the iron-binding peptide TPEVDDEALEK (125–135 AA) from β -LG was found to be transported across the intestinal barrier (Picariello et al., 2013). Whether any of the other whey peptides are antioxidant has yet to be determined.

DO WHEY PRODUCTS ACT AS ANTIOXIDANT PROTECTOR IN VIVO?

Although physiological biomarkers are limiting, human or animal intervention trials with diets that include whey products are the best assessment of antioxidant benefit. Table 5 summarizes the most recent animal trials that tested the antioxidant effect of WP rich diets. Bounous et al. (1989) proposed that a diet rich in GSH AA precursors, such as Cys, would boost cellular GSH production. As WP are Cys-rich, Bounous et al. (1989) fed elderly (17–20 mo old) C7BL/6NIA male mice a diet rich in WPC (20 g/100 g of diet) for 3 mo. Animals were euthanized and GSH levels in liver and heart were measured. Mice on WPC diets had significantly higher GSH levels in liver (9 μ mol GSH/g of liver) and heart (1.6 μ mol of GSH/g of heart) than those animals fed a casein-rich diet (20 g/100 g of diet) or a control chow diet (8 μ mol of GSH/g of liver and 1.3–1.5 μ mol of GSH/g of heart; $P < 0.05$) for the same time period. In addition, the WP-rich diet appeared to extend the lifetime of the aged mice, with a 55% mortality rate reached at 125.0 ± 41.6 d compared with 92.2 ± 55.2 and 92.7 ± 31.7 d for mice fed casein-rich or chow diets, respectively ($P < 0.05$). Liver GSH was also increased in Fisher rats fed with WP (150 g/1,000 g of diet; 55 μ mol of GSH/mL of tissue extract) during 8 wk compared with those on a casein-rich diet (44 μ mol of GSH/mL; Haraguchi et al., 2011). Interestingly, a diet supplemented with 10% whey powder protected Wistar rats against induced CCl_4 hepatotoxicity (Ashoush et al., 2013). Ashoush et al. (2013) proposed that this protection was as a result of an increase in total GSH plasma levels (CCl_4 plus WP = 16.74 ± 1.2 mg/dL vs. CCl_4 = 9.94 ± 0.84 mg/dL). As a model of oxidative stress,

Sprague-Dawley rats were fed a diet high in iron (2,000 mg/kg) for 6 wk. Those animals that also received 10% WP had increased GSH in blood erythrocytes (11.43 ± 0.71 μ M) compared with controls (GSH = 8.75 ± 0.71 μ M; Kim et al., 2013). However, CAT levels were not significantly increased after WP supplementation (Kim et al., 2013). In agreement, a combination of exercise and WP intake over an 8-wk test period had little effect on liver CAT activity in Fisher rats fed a WP-rich diet (150 g/1,000 g) compared with those on a casein-rich diet (30 U/mg of protein; Haraguchi et al., 2011). In contrast, Athira et al. (2013) observed a significant increase in liver CAT levels in Swiss albino mice who received an intraperitoneal injection of WP hydrolyzed by subtilisin (4 mg/kg of BW; CAT = 193.66 ± 18.61 U/mg of protein) compared with mice without WP administration (149.67 ± 12.83 U/mg of protein). All of the Swiss albino mice in their study received paracetamol orally (300 mg/kg of BW) for 2 d to induce oxidative stress before WP administration (Athira et al., 2013).

In a human intervention study, blood GSH levels were evaluated over 6 wk in 18 male participants subjected to strenuous aerobic training and a dietary supplement of 1 g of WPI/kg of body mass per day. Blood GSH levels were significantly lower in those subjects who performed exercise than those who did not ($P < 0.05$). The addition of WP supplementation to an exercise regimen prevented this GSH depletion in blood (Middleton et al., 2004). In agreement, Sheikholeslami Vatani and Ahmadi Kani Golzar (2012) observed increased GSH plasma levels in 30 overweight young men who consumed WPI and performed resistance training for 8 wk (173 ± 22 nM GSH/L vs. control group, 144 ± 20 nM GSH/L; $P < 0.05$). Levels of GSH in plasma were also increased by 23% in steatohepatitis patients who received 20 g of WPI/d for 12 wk compared with GSH levels before WPI supplementation (Chitapanarux et al., 2009). In another human trial, 31 subjects received 15 to 45 g of pressurized WPI/d over a 2-wk period. The GSH levels in lymphocytes extracted from blood were 24% higher after 45 g of WPI consumption than participants who did not consume WPI (Zavorsky et al., 2007). In contrast, blood GSH levels remained unchanged over the 4-h sampling period in male subjects who received an acute dose of WPI (0.8–1.6 g of WPI/kg of BW) (Middleton et al., 2004). Measuring levels of GSH in plasma is one of the most common techniques to detect whey product antioxidant protection in vivo. A positive correlation exists between low plasma GSH levels and disorders in which oxidative stress is a contributing factor, such as cardiovascular disease (Shimizu et al., 2004), polycystic ovary syn-

Table 5. Antioxidant effect of whey in vivo

Product ¹	Protein pretreatment	Food matrix	Subjects	Dosage	Time	AOX parameters ²	Results ³	Reference
WPI (InPro 90)	Pressurized	Bars (17 g protein/bar), orally	Men and women (31)	15, 30, or 45 g/d	2 wk	GSH in lymphocyte	30 and 45 g/d ↑ 24% GSH	Zavorsky et al. (2007)
Whey-derived peptide (NOP-47)		Powder in water, orally	Men and women (20)	5 g peptide/d	2 wk	FRAP and oxidative stress in plasma	15 g/d = GSH FRAP value (375 ± 80 μmol of TE/L, preingestion: 354 ± 82 μmol of TE/L) ↑ Plasma total AOX capacity (61%) compared with baseline ↑ Plasma GSH (28%) compared with baseline GPx ↑ in whey diet (39.9 ± 4.8 U/g Hb) compared with casein (26.2 ± 6.7 U/g Hb)	Ballard et al. (2009)
WPI		Powder in water, orally	Patients (38) with nonalcoholic steatohepatitis	20 g/d	12 wk	ABTS Plasma glutathione level	↑ Plasma total AOX capacity (61%) compared with baseline ↑ Plasma GSH (28%) compared with baseline GPx ↑ in whey diet (39.9 ± 4.8 U/g Hb) compared with casein (26.2 ± 6.7 U/g Hb)	Chitapanarux et al. (2009)
WPX (Peptamen 1.5)	Hydrolyzed	Enteral formula	25 elderly men and women with ischemic stroke	1.2 g protein/kg/d	5 d	GPx in blood	GPx ↑ in whey diet (39.9 ± 4.8 U/g Hb) compared with casein (26.2 ± 6.7 U/g Hb)	de Aguiar-Nascimento et al. (2011)
WPX		ND	Rats (32) + resistance exercise	150 g protein/kg	8 wk	GSH, CAT in liver Lipid peroxidation in liver and muscle	After exercise: WP ↑ GSH ↓ Lipid peroxidation compared with casein diet No differences in CAT	(Haraguchi et al., 2011)
WPC		Powder in water, orally	Rats (80, male), CCl ₄ -induced hepatotoxicity	50 mg/d	30 d	Total AOX activity in liver Lipid peroxidation in liver	↑ AOX activity ↓ lipid peroxidation	Gad et al. (2011)
WPI		Powder, orally	Overweight men (30) + resistance exercise	30 g/d	6 wk	GSH in blood	Whey diet and exercise GSH ↑ compared with control: 173 ± 22 nm/L Control = 144 ± 20 nm/L Placebo = 163 ± 26 nm/L	Sheikholeslami Vatani and Ahmadi Kani Golzar (2012)
Fresh whey	Preheating 70°C, 10 min hydrolyzed by subtilisin (pH 8.0–9.0, 55–60°C, 8 h)	Orally i.p. injection	Mice (24, male), paracetamol-induced oxidative stress	Oral: 8 mg/kg Injection: 4 mg/kg	4 d	Serum oxidative biomarkers (GPT, ALP, creatinine, BUN) CAT, SOD, GPx Lipid peroxidation inhibition	↓ Oxidative biomarkers and lipid peroxidation ↑ CAT, SOD and GPx	Athira et al. (2013)
WPX		Powder	Rats (40, male), carbon tetrachloride (CCl ₄)-induced hepatotoxicity	10% whey	28 d	GSH in plasma Lipid peroxidation inhibition	↑ GSH levels (CCl ₄ plus WP = 16.74 ± 1.2 mg/dL, CCl ₄ = 9.94 ± 0.84 mg/dL) ↓ Lipid peroxidation	Ashroush et al. (2013)

Continued

Table 5 (Continued). Antioxidant effect of whey in vivo

Product ¹	Protein pretreatment	Food matrix	Subjects	Dosage	Time	AOX parameters ²	Results ³	Reference
Fresh WP		Pellet, orally	Rats (30 male), iron overload-induced oxidative stress	10 g of protein/100 g	6 wk	Plasma radical trapping potential GPx, CAT, SOD, GST, GSH	↑ Plasma AOX activity, SOD and GSH in erythrocytes No differences in GPx, CAT and GST ↓ Lipid peroxidation and DNA damage	Kim et al. (2013)
Whey peptides	Commercial peptides	Orally with gastric tube	Mice (28, male) UV B radiation	400 or 800 mg of peptide/kg	17 wk	DNA damage peroxidation DNA oxidative damage (detection of 8-OHdG ¹)	Prevention of DNA damage caused by radiation	Kimura et al. (2014)
WPI	Chymotrypsin (pH 7.0, 37°C, 2 h) followed by filtration (30, 10 and 5 kDa cut-off)	Liquid, orally	Mice (140) + exercised-induced fatigue	1.5 g of protein/kg/d	6 wk	SOD, GPx in mitochondria and gastrocnemius	Mitochondria: SOD and GPx ↑ with WPH <5kDa (SOD: 549.20 ± 19.08 U/mg protein, Control = 388.41 ± 34.56 U/mg protein) and WPH5–10 kDa Gastrocnemius: no differences in SOD GPx ↑ with WPH <5 kDa and WPH 5–10 kDa AOX activity ↑ 23% from baseline	Liu et al. (2014)
Mixture of MPC, commercial WPH (DH = 32%, 78% protein; DH = 45%, 75% protein)	Hydrolyzed	Liquid, orally	Women	0.3 g/kg	Acute ingestion	ORAC in plasma		Power-Grant et al. (2016)

¹WPI = whey protein isolate; WPX = whey protein, type not specified; WPC = whey protein concentrate; MPC = milk protein concentrate; WPH = whey protein hydrolysate; DH = degree of hydrolysis.

²AOX = antioxidant; GSH = glutathione; GPx = glutathione peroxidase; CAT = catalase; GPT = glutamic-pyruvic acid transaminase; ALP = alkaline phosphatase; BUN = blood urea nitrogen; SOD = superoxide dismutase; GST = glutathione S-transferase; 8-OHdG = 8-hydroxy-2'-deoxyguanosine.

³TE = Trolox equivalents.

drome (Murri et al., 2013), and autism (Frustaci et al., 2012). However, plasma GSH levels are unlikely to reflect intracellular GSH in target organs such as the liver, brain, or muscle routinely exposed to oxidative stress (Ballatori et al., 2009). In contrast, charting the oxidation levels of particular proteins involved in disease onset and progression would provide more relevant biomarkers in cellular assays and dietary intervention trials (Friehoff et al., 2015).

CONCLUSIONS

Bovine whey and individual WP exhibit antioxidant activity. This bioactivity is observed with different commercial whey products (WPI, WPC), is relatively resistant to processing method, and is increased by enzymatic hydrolysis. Subtilisin appears the enzyme of choice to deliver the most potent whey hydrolysate fractions. Several synthetic peptides derived from β -LG and α -LA have demonstrated antioxidant activity. It is also likely that free AA released during hydrolysis contribute to this bioactivity. It is important to note that readouts are conflicting from different antioxidant methodologies, different whey products, different dosages, and in the translation of cellular assays to plasma biomarkers. Exposing cell lines to whey test samples directly results in an increase in GSH levels; however, the biological relevance of these experiments is questionable. Whether the bioactivity survives gut transit, passes through the intestinal barrier, and reaches its target cells will ultimately determine its efficacy as a dietary antioxidant ingredient. Certainly, some evidence suggests that WP supplementation alters plasma biomarkers for antioxidant activity, especially in individuals exposed to high oxidative stress levels, either from illness or intense exercise. However, the number of participants in these studies is small and the relevance of these biomarkers to target organs exposed to oxidative stress requires further investigation. The antioxidant potency of whey products is lower than well-known plant antioxidants, such as green tea, although WP can be added to food at much higher concentrations. Future studies should focus on the synergistic or antagonistic effect of novel combinations of whey products with other known antioxidants within food matrices if whey products are to deliver antioxidant benefits.

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